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Expression, purification, and large-scale production of the human recombinant Annexin-V protein

Gayatri Suresh Kumar
University of Arkansas, Fayetteville

This thesis is approved.

Thesis Advisor:

Thesis Committee:

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Expression, Purification, and Large-Scale Production of the Human Recombinant Annexin-V Protein

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Gayatri Suresh Kumar

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ABSTRACT

Annexin-V is a cytosolic protein involved in many significant physiological roles including stabilization of the phospholipid membrane, regulation of blood coagulation, and cell apoptosis or programmed cell death. Though the exact mechanism of Annexin-V in the human body is not fully understood**,** its wide range of applications in the medical field cannot be denied. Annexin-V can be applied in cancer therapy as a diagnostic to detect cell apoptosis and determine the effectiveness of chemotherapeutic drugs. Furthermore, Annexin-V could even be used as a drug delivery vehicle in the treatment of cancer. Commercially useful proteins like Annexin-V are in high demand, however, one big obstacle is that yielding a large amount of pure Annexin-V could be a tedious and expensive procedure. Performing research with this protein could also become a huge financial undertaking. The focus of this project was to study the optimal conditions by which the Annexin-V protein could be expressed**,** purified**,** and mass-produced. An additional goal was to devise a cost-effective procedure of mass-producing Annexin-V. SDS-PAGE was the primary method applied to test the intensity and purity of the Annexin-V sample yielded from experiments. SDS-PAGE results establish that the *E. coli* over-expressed the Annexin-V protein. Purification using an ion exchange (DEAE) column yielded Annexin-V of relatively high purity; a step-wise salt gradient was significantly more effective than a linear salt gradient in eluting a more pure product from the column. A bioreactor was used to perform batch fermentation. Three types of media were used for the batch experiments: LB broth only, LB and 9 g/L glucose, and M9+glucose media consisting of M9 salts, trace elements, biotin, and 9 g/L glucose. Among the batch fermentations, LB and 9 g/L glucose yielded the highest amount of Annexin-V. However, the E. coli were not able to successfully grow in the M9 media mixture. Additional experiments using the M9 media

will have to be performed to verify this observation. The data collected throughout this project suggest that: 1) *E. coli* cultures are effective in over-expressing Annexin-V, 2) A DEAE purification column, accompanied by the use of a step-wise salt gradient, yields Annexin-V of considerably high purity 3) The use of LB/glucose media is an effective method to mass-produce Annexin-V.

INTRODUCTION

Annexin-V is a member of a family of cytosolic proteins (Annexins) that have structural similarities and are known to effectively bind to the phospholipid membrane. A key property that all the proteins in the Annexin family share is their ability to bind to calcium. In fact, Annexins are calcium-dependent proteins in that their binding affinity to the surface of phospholipids is significantly reduced without the presence of calcium.[3] In addition to their affinity to calcium, they all preferentially bind to anionic phospholipids and have molecular weights of 35-40 kDA; Annexin-V has a molecular weight of 36 kDa.[7] Annexins consist of repeats of individual amino acid domains that contribute to their high affinity to calcium and phospholipid ligands.[8]

Annexin-V is involved in many physiological roles including binding to the phospholipid bilayer membrane and maintaining its integrity. Other roles include involvement in the processes of endocytosis and exocytosis, enzyme inhibition, and anticoagulation.[6] In particular, Annexin-V, discovered in 1985, was first mainly known as a regulator of blood coagulation.[3,4] The Annexin-V protein is used in many scientific experiments because the crystal structure of the human recombinant Annexin-V protein has been established.[1] The crystal structure of Annexin-V is important because it facilitates the study of the phospholipid membrane-binding property of the Annexin family of proteins. Another important reason that Annexin-V is a protein of great interest

to researchers is because it plays a huge role in cell apoptosis.[5] Due to the involvement of Annexin-V in the process of cell apoptosis, it has a wide array of applications in the fields of biotechnology and pharmacy. Annexin-V attaches to the outer surface of cells undergoing apoptosis. Hence, it is relatively easy to image the apoptotic cell if using fluorescently-labeled Annexin-V. This technique of using radioactively-labeled human recombinant Annexin-V is very common, and is widely used in clinical trials to image "ischemic injury, transplant rejection, and the response of tumors to chemotherapeutic agents".[2] Annexin-V has also been proven to be useful as a mechanism of drug delivery in the treatment of chronic diseases like cancer.

With the vast number of important physiological roles that Annexin-V is associated with, the production of pure Annexin-V is in great demand. The production of a protein requires the use of host cells. *E. coli* are often regarded as an effective host to produce recombinant proteins. This is due to several factors: 1) The genetics and physiology of *E. coli* has been greatly studied, and the manipulation of *E. coli* to yield recombinant DNA products has been widely applied, 2) It has been demonstrated in many previous studies that expression of recombinant proteins using *E. coli* could yield product levels that comprise of approximately 30 to 50% of total cellular protein, 3) *E. coli* cultures are ideal for large-scale production of recombinant proteins because it can yield high cell concentrations of over 100 g/L.[9] There are a few disadvantages to using *E. coli* to produce proteins like Annexin-V. These include the possible requirement of a large oxygen supply, as well as the accumulation of metabolic by-products which could potentially inhibit *E. coli* growth. However, the benefits of using *E. coli* to produce recombinant proteins like the human recombinant Annexin-V greatly outweigh the disadvantages.

Using *E. coli* as an expression system would not be possible if it were not for the development of genetic engineering or specifically recombinant DNA technology. The primary

purpose of recombinant DNA technology is to genetically modify microorganisms such as *E. coli* by implanting particular genes into them so that they can produce a useful product such as Annexin-V. Recombinant DNA technology using *E. coli* requires the following steps: 1) The DNA fragment coding for the protein of interest is synthesized or isolated, 2) An *E. coli* plasmid is isolated and restriction endonucleases are used to cleave specific sites in the plasmid, allowing the DNA fragment of the protein of interest to be inserted, 3) The recombinant DNA molecule is directly introduced into *E. coli* cells via a process known as transformation, 4) The host cells that have incorporated the recombinant DNA molecule are selected, cloned, and maintained to be used in cultures for expression of the protein of interest.[10]

In order for proteins like Annexin-V to be commercially-applicable, they need to be purified. There are many techniques used to purify proteins, with affinity chromatography and ion exchange chromatography being frequently used ones. Polyhistidine tags or glutathione tags are often used in an affinity chromatography procedure to purify proteins such as Annexin-V. The general purpose of purification is to isolate the protein of interest from contaminant bacterial proteins. There is a specific way in which affinity chromatography achieves this purpose. In order to perform affinity chromatography, the protein of interest should already be tagged. In addition, this procedure requires the use of a purification column with resin beads that would bind the tagged protein. A concentrated solution of the tagged protein of interest is allowed to flow through the column. At this time, high affinity binding occurs between the tagged protein and the resin. Meanwhile, most of the contaminant proteins will be eluted out of the purification column. In order to displace the protein bound to the resin and elute it out of the purification column, the column has to be washed with an excess amount of an eluting buffer that can accomplish this task.

Typically, increasing concentrations of sodium chloride or imidazole are used to serve this purpose.[11]

Ion exchange chromatography is similar to affinity chromatography in that a purification column with resin is necessary. Ion exchange is a protein purification procedure that is based on reversible adsorption of charged molecules; usually, protein separation is achieved by using weak exchangers or exchange resins that can bind to the charged protein of interest. In the case of Annexin-V, which has an overall negative net charge, an anion-exchanger is most effective. A DEAE-sepharose column, a purification column with anion exchanger dietylaminoethyl (DEAE) is often used to purify anionic proteins like Annexin-V. DEAE-sepharose is a resin that is essentially a matrix of chemically-modified and cross-linked agarose bead ions. DEAE-sepharose is positively charged at neutral pH.[12]

In order for researchers and doctors to be able to use Annexin-V in clinical experiments, and for pharmaceutical industries to take advantage of its medical uses by manufacturing it, large-scale production of the Annexin-V protein is necessary. But, large-scale production is often a very expensive procedure. Therefore, it is necessary to first determine which type of method is the most cost-effective way of mass-producing the protein of interest. A bioreactor is the device typically used to grow cultures for large-scale production of proteins. It can be operated in three different modes: batch, fed-batch, and continuous. There are four important phases associated with growth cultures: lag, log, stationary, and death. The culture in the bioreactor first goes through the lag phase during which the cells are trying to adjust to the new environment and begin synthesizing enzymes required to utilize the nutrients in the medium. Subsequent to this, the cells go through exponential growth during the log phase. In the stationary phase, the cells begin to exhaust the amount of nutrients necessary for their growth. And finally, in the death phase, there is a decrease

in cell number. In a batch process, a cell culture is added to a sterile nutrient medium, and the host cells (for example, *E. coli*) are allowed to grow in the bioreactor. There are not any other additions to the culture, and the process is typically terminated during the stationary phase. A fed-batch process is similar to a batch process except there is controlled addition of nutrient medium. Typically, industrial processes manufacturing antibiotics use fed-batch processes because controlling the supply of nutrients (which can be accomplished by varying the feed rate, for example) allows for greater control of cell growth. In addition to the feed rate, other variables can varied such as the pH and the dissolved oxygen level. Fed-batch processes are also sometimes preferred because it is economically practical; the total mass of culture increases during fed-batch operation, and this usually correlates to greater yield of the protein of interest. In a continuous process, the amount of nutrients necessary for cell growth is continuously fed into the bioreactor; a certain amount of culture is also continuously withdrawn from the bioreactor. Other than factors such as the mode of operation, temperature, pH, feed rate, and the dissolved oxygen level, the type of nutrient medium used could also be experimented with. Varying such factors could help in optimizing the yield of the protein of interest, and in determining a cost-effective procedure to produce the protein.[13]

MATERIALS AND METHODS

Expression of Annexin-V using E. coli:

An overnight culture was prepared by swirling one colony of *E. coli* containing the Annexin-V gene in 5 mL of LB media. About 12 µL of 50 mg/mL ampicillin was added to the culture. The strain of E. coli used was engineered to be ampicillin-resistant so addition of ampicillin prevents the growth of contaminant microorganisms. The overnight culture was then placed in the

incubator-shaker at 37ºC. The following day, the overnight culture was added to 100 mL of LB media and approximately 200 µL of ampicillin. The optical density of the E. coli culture was periodically checked using a Beckman Coulter DU 800 spectrophotometer at a wavelength of 600 nm. After the *E. coli* culture reached an optical density of about 0.3-0.4, IPTG, isopropyl-beta-D-thiogalactopyranoside, to induce expression of Annexin-V. After approximately three hours, the cell culture was centrifuged at a speed of approximately 4200 rpm for 45 minutes. A small sample of the supernatant was collected in an Eppendorf tube, while the rest of the volume was decanted. The collected sample as well as the pellet was stored in a -80ºC freezer until analysis using SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). On the day in which SDS-PAGE is performed, a gel was prepared for the analysis of the samples collected during expression. Blue dye with beta-mercaptoethanol was added to all the samples, and heated. The samples, as well as a molecular protein marker were then loaded onto the gel. Subsequent to this, SDS-PAGE was initiated at a voltage of 200 V. After staining and de-staining the resulting gel, it was checked for expression of Annexin-V.

Purification of Annexin-V using DEAE-sepharose column:

Prior to purification of Annexin-V, the *E. coli* pellet obtained after expression was pre-treated. The pellet was re-suspended in cold 50 mM Tris HCl, 10 mM $CaCl_2$ at pH 7.2. The pellet slurry, placed in an ice bath, was sonicated to yield a homogeneous pellet mixture. Then, the pellet slurry was centrifuged for 20 minutes at 4ºC and a speed of 4200 rpm. After collecting a small amount of the supernatant for analysis, and discarding the rest, the pellet was re-suspended in 50 mM Tris HCl 20 mM EDTA at pH 7.2. The EDTA causes the Annexin-V protein to be released from the pellet into the supernatant. This pellet slurry was again centrifuged at the same conditions as stated above. The supernatant collected after centrifugation was dialyzed in 20 mM Tris HCl at pH 8.0

and at 4ºC. The next day, the DEAE-sepharose column was set up and first equilibrated with a buffer that is a 1:10 dilution of the dialysis buffer. Then the dialyzed supernatant was pumped into the purification column. The purification column was connected to a chromatograph to visually detect peaks corresponding to the amount of protein eluted. Soon after the Annexin-V dialysis buffer is pumped into the column, a large contaminant peak should be observed. This is when a salt gradient was used to elute the Annexin-V protein out of the purification column. Both a linear salt gradient as well as a step-wise salt gradient were used. To establish a linear salt gradient, a gradient salt mixer was applied. For the step-wise gradient, increasing concentrations of sodium chloride solution were used: 100 mM NaCl, 200 mM NaCl, 300 mM NaCl, 400 mM NaCl, 500 mM NaCl, and 1 M NaCl. Each salt solution was individually pumped into the column, starting from 100 mM NaCl. If a peak is observed on the chromatograph, it is indicative of some of the Annexin-V being eluted from the resin in the purification column. Hence, samples were collected whenever peaks were observed. Guanidinium hydrochloride was pumped into the column as well, and a sample was collected. All the collected samples were stored in -80ºC until SDS-PAGE analysis. The purification column itself, at the end of the purification experiment, was regenerated using milliQ water and 20% ethanol.

Fermentation using a Bioreactor:

A 5 mL overnight culture was prepared similar to the first step of the Annexin-V expression experiment. A 100 mL pre-culture was prepared the following day. The bioreactor was sterilized for use the day after. A 1 L medium with the 100 mL pre-culture from the previous day was prepared for *E. coli* growth in the bioreactor. A bioreactor with an ADI 1010 bio controller and an ADI 1025 bio console was adjusted at a rotor speed of 700 and a temperature of 37ºC. Air was continuously supplied to the bioreactor. The optical density of the *E. coli* culture was frequently

checked to see if a value of 0.3 or 0.4 was reached, at which time the culture was induced with 3 mM IPTG. After a 3-hour induction period, the operation of the bioreactor was terminated, and the cell culture was centrifuged at a temperature of 4ºC and a speed of 4200 rpm. The resulting pellet was stored in -80ºC until SDS-PAGE and densiometry were performed.

RESULTS

The SDS-PAGE results from the expression of Annexin V, shown in Figures 1 and 2, established that Annexin-V has been successfully expressed by the *E. coli*. Experiments performed in the Beitle laboratory have indicated that the optimum IPTG concentration to add to the *E. coli* culture is 3 mM, and the ideal induction time is approximately 3 hours.

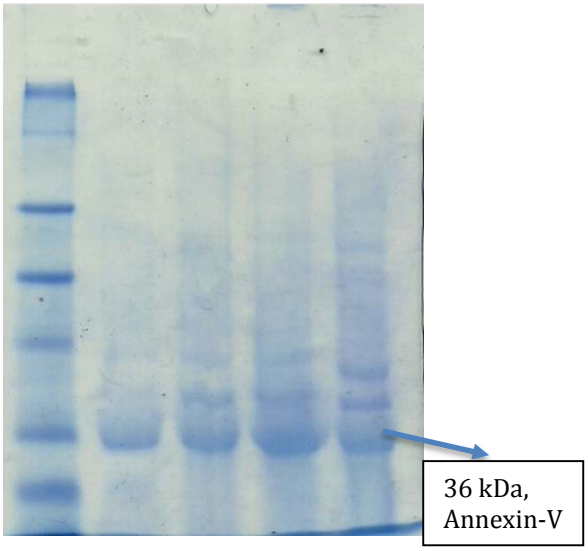

Figure 1. Expression SDS-PAGE
1: Molecular Marker
2: Supernatant
3: Pellet
4: Induced pellet
5: Un-induced pellet

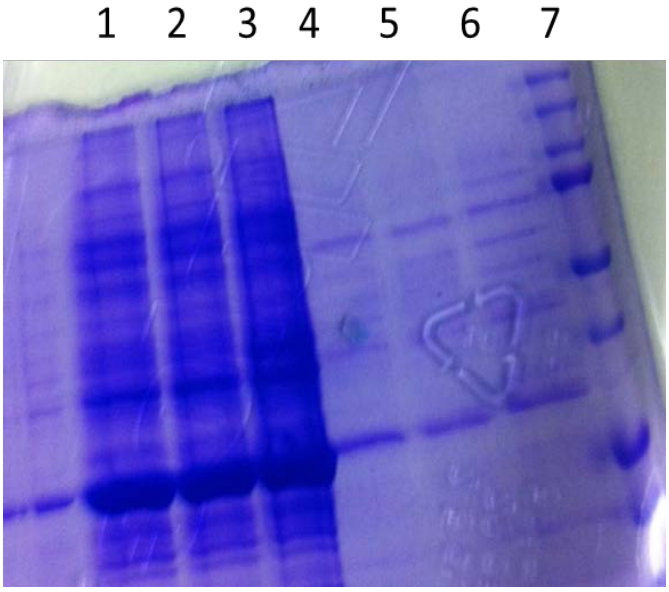

Figure 2. Expression SDS-PAGE
1: 10 mM induced (pellet)
2: 10 mM induced (supernatant)
3 3 mM induced (pellet)
4: 3 mM induced (supernatant)
5: Un-induced (pellet)
6: Un-induced (supernatant)
7: Protein Marker

As Figure 3 demonstrates, as expected, the EDTA solution caused the Annexin-V protein to be released into the supernatant from the pellet. Purification of Annexin-V was performed using the DEAE-sepharose column, and applying two different techniques to elute the protein from the column: linear salt gradient and step-wise salt gradient. The SDS-PAGE results for the linear salt gradient are shown in Figure 4, while those for the step-wise salt gradient are shown in Figure 5. Though both techniques yielded reasonably pure Annexin-V, the latter was more effective. The SDS-PAGE from the purification experiment using a step-wise salt gradient demonstrates that virtually all the Annexin-V protein is eluted out of the purification column at a NaCl concentration of 200 mM. Visually, it appears that purification via a DEAE column and a step-wise salt gradient yields Annexin-V with a purity of over 90%.

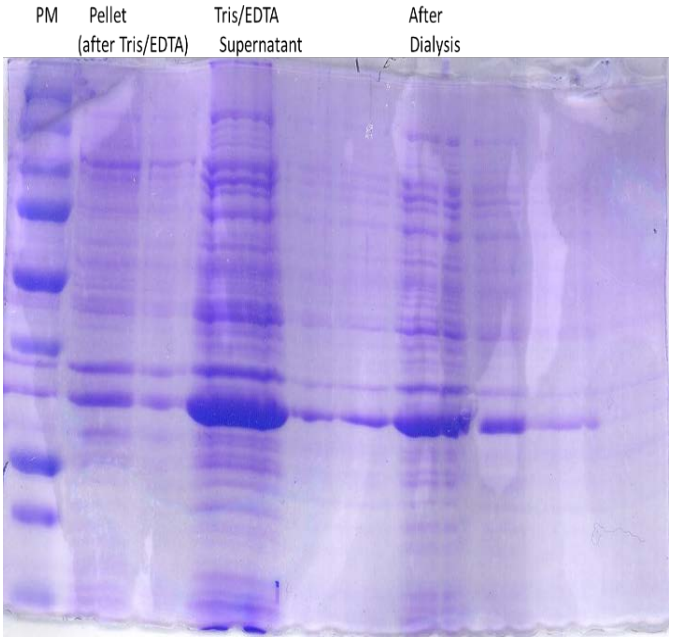

Figure 3. Tris/EDTA and Dialysis
(PM indicates “Protein Marker”)

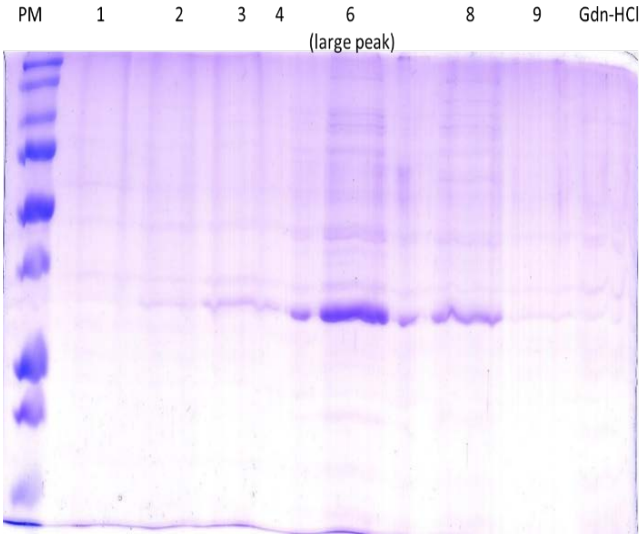

Figure 4. Purification SDS-PAGE with Linear Salt Gradient (Gdn-HCl indicates "Guanidinium hydrochloride)

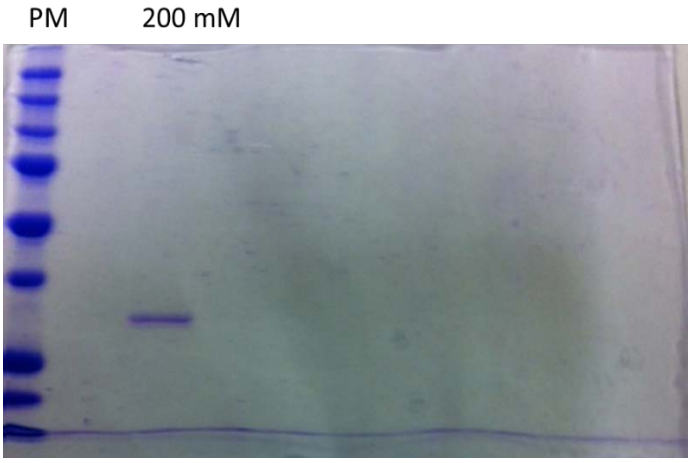

Figure 5. Purification SDS-PAGE with Step-wise salt gradient (200 mM indicates 200 mM NaCl)

Batch fermentation experiments were performed using the bioreactor. The parameter that was varied was the type of medium used. Three different types of media were used: LB medium, LB medium plus glucose, and M9 salts+glucose medium. Annexin-V was successfully mass-produced in LB medium and LB plus glucose medium. According to the results from the densiometry test

using the SDS-PAGE shown in Figure 6, the protein yield in LB medium was 28%, while it was approximately 16% greater in the LB+ glucose medium.

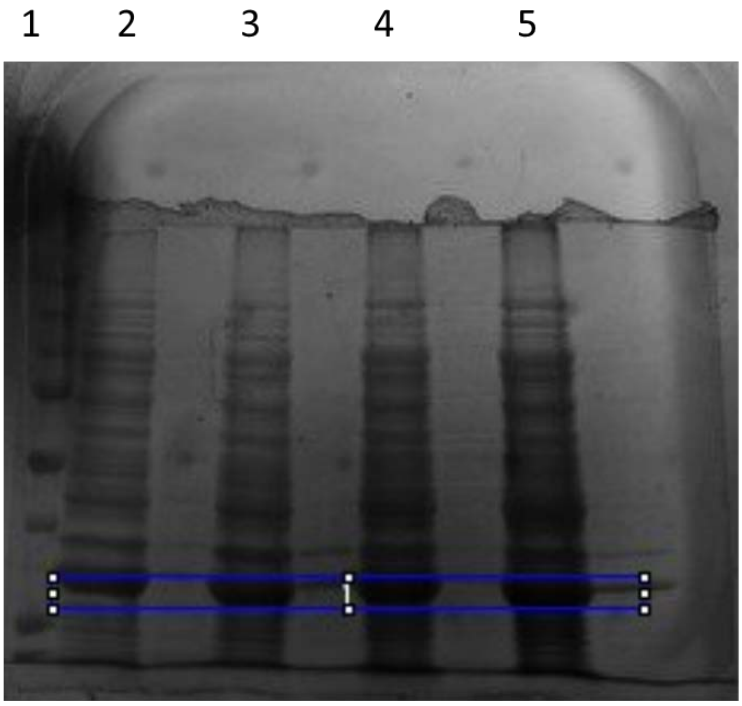

Figure 6. SDS-PAGE Batch Fermentation
1: Protein Marker
2: LB Medium $CaCl_2$ Pellet
3: LB Medium EDTA Pellet
4: LB+9 g/L glucose $CaCl_2$ Pellet
5: LB+9 g/L glucose EDTA Pellet

Figure 7. Densiometry Plot Peaks from left to right correspond to lanes 2-5 in SDS-PAGE shown above

DISCUSSION

The data collected from the experiments performed establish that an *E. coli* culture induced with 3 mM IPTG successfully expresses the Annexin-V protein. The DEAE-exchange column is an effective purification column for the purification of Annexin-V. This column was used because it allows for a simple purification protocol since it functions primarily based on the opposite charges of the DEAE-resin and the Annexin-V protein. The step-wise salt gradient was quite successful in the goal of yielding Annexin-V of significantly high purity. In the future, the purification experiments using glutathione or polyhistidine-tagged Annexin-V will be performed in the Beitle laboratory to determine whether a more optimal protocol could be applied with regard to producing Annexin-V of higher purity. Mass-production of Annexin-V using *E. coli* cultures grown in a bioreactor can be achieved. LB plus 9 g/L glucose medium yielded a greater amount of Annexin-V compared to just LB medium. This is consistent with common knowledge because glucose acts as an additional source of nutrients for the *E. coli*, effectively allowing it to produce more of the Annexin-V protein. Additional experiments using the M9 salts+glucose medium will be performed to evaluate whether this medium can be used to mass-produce Annexin-V. Fed-batch fermentation will also be performed in the Beitle laboratory to determine if this procedure offers a significantly higher amount of Annexin-V compared to batch fermentation. Another goal of the Beitle laboratory with regard to the continuation of this project is to perform several different biophysical experiments to characterize the Annexin-V protein including thermal denaturation, circular dichroism, trypsin digestion, isothermal titration calorimetry, NMR spectroscopy, and tests to determine the apoptotic activity of the Recombinant Annexin V.

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